PECTIN RELATIONS OF SCLEROTINIA CINEREA[1]

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The pectic substances which constitute the middle lamellae of fleshy fruits, and thus serve as the cementing material between cells, are of great interest to the plant pathologist because of the fact that if an invading organism is to make its way into a tissue, it must either pass along the line of the middle lamella *between* the cells, or it must bore *through* the cells. In the first case a pectic enzyme at the hands of the parasite may be presupposed, and in the second case a cellulose enzyme.

The mechanism of infection by *Sclerotinia cinerea* has received particular attention recently by COOLEY (4) and by VALLEAU (8). COOLEY maintains that its hyphae in plum tissue are mainly intracellular; while VALLEAU finds them to be entirely intercellular, and produces photomicrographs to prove it. COOLEY could not demonstrate the presence of an enzyme which would dissolve the calcium pectate of the middle lamella. He also failed to find any evidence of a softening of the tissue in advance of penetration. VALLEAU, by more careful methods, noticed a marked separation of the cells in rotted tissue, and demonstrated that an extract of rotted apple would bring this about. The agent in this case is the enzyme designated by VALLEAU "pectinase," but more correctly called "pectosinase" according to the terminology of its discovery (3, 10), and of ATKINS (2). VALLEAU's illustrations show that it is secreted considerably in advance of the penetrating hyphae. The question of oxalic acid being the solvent was considered by both writers. COOLEY found appreciable amounts of the acid in rotted peaches and plums, but was not convinced that it was the middle lamella solvent. VALLEAU demonstrated that dilute solutions of oxalic acid would soften the tissues of these fruits, but not of potato, which facts convinced him that this acid is not the only factor involved in the disintegration of the middle

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lamella. Both writers agree that *Sclerotinia* is more virulent on ripe than on green fruit, because in the ripe fruit the middle lamellae are disintegrated and allow easier penetration.

COOLEY found that the fungus coagulates soluble pectin, even when calcium is presumably absent from the medium, and that it does not dissolve precipitated "calcium pectinate" suspended in an agar nutrient medium. As to the question whether any pectic material is assimilated by this fungus, HAWKINS (**6**) found no diminution in the pentosan content of peaches which had been rotted by it, and COOLEY's observation that it could not grow on "calcium pectinate" would indicate failure to assimilate this pectic material, at least.

The present status of the question of pectin relations of *Sclerotinia cinerea*, therefore, is that it disintegrates the tissues by dissolving the middle lamella with a secreted pectosinase, and probably also with oxalic acid. What disposition is made of the dissolved pectic material, and what function the pectase serves, have not been suggested. The fungus also coagulates soluble pectin by means of pectase. Pectinase (see later for definition) has not been demonstrated.

During the course of some work on *Sclerotinia* the writer (**9**) made several interesting observations on the behavior of this fungus toward the various pectic substances. These are recorded in the following notes.

Assimilation of pectin

Before recounting the observations made on the pectic relations of this fungus it might be well to explain the nomenclature that has been adopted in this paper. It is the same as that used by ATKINS (**2**). Pectosinase is the enzyme that dissolves the middle lamella, with the formation of soluble pectin. Pectase coagulates soluble pectin to a gel in the presence of a calcium (or barium or strontium) salt. Pectinase hydrolyzes soluble pectin, and also the gel formed by pectase, to reducing sugars. Such definite names have not been given to the various pectic products; hence the particular one in question in any particular case can be told only by the context.

In order to test the assimilatory power of *Sclerotinia* toward soluble pectin, a sample of the latter was made by precipitating prune juice with alcohol, reprecipitating twice, and then drying in an oven and grinding. The resultant powder was incorporated in a mineral nutrient medium to the extent of 0.4 per cent. With no sucrose added, growth took place, but only about one-fifth as rapidly as when some sucrose was present. Whether this growth was due to the assimilation of pectin, or to sugars adsorbed during the preparation of the pectin, was not evident, since no analyses were made. To test this point quantitatively, another preparation of pectin, from peach, was made. It was decided to follow the product by analyzing for its content of pentosan. Although this is open to many objections, the chief of which is the questionable accuracy of the existing methods of pentosan determination, no other way of studying the fate of the pectin in media appeared feasible. The official (1) phloroglucide method was used. The pectin preparation showed a content of 15.0 per cent furfural, or 25.6 per cent pentosan. SCHRYVER and HAYNES (7) obtained 18.6 per cent furfural in turnip pectin. They had a far purer preparation, however, than the writer attempted to obtain.

A medium containing mineral salts and 2 per cent of pectin was prepared, and two Erlenmeyer flasks, each with 50 cc. of medium, were sterilized and inoculated with spores of the fungus. Growth was slow but typical. The medium became a gel, due to the coagulation of the pectin by the fungus. After three weeks the colony in each flask had attained a diameter of 3.5 cm. (see 9 for the writer's method of estimating the growth of this fungus). By this time also the gel had become almost completely liquefied, and the liquid portion gave no precipitate with alcohol. The colonies were carefully removed, freed from the adhering gel of the medium as well as possible, and then the two mycelia and the two media combined for analysis. The results were as follows:

Pentosan in original media	0.502 gm.
Pentosan in mycelia of culture 572	0.014 gm.
Pentosan in residual media	0.040 gm.

Thus a total of 0.054 gm. of pentosan remained in the culture flasks at the end of the period of growth, whereas 0.502 gm. was

present in the pectin at the start. Very evidently during the growth of the fungus the pectin was assimilated in part, at least. About nine-tenths of the furfural-yielding material of the pectin had disappeared. Presumably this material was utilized by the fungus for energy, but was not stored in any appreciable quantity in the hyphae. It is possible that the small amount found in the latter was due to pectic gel enmeshed in the hyphal weft.

The course of the changes involved when *Sclerotinia cinerea* grows on a pectin medium may be outlined as follows. There is first a coagulation of the soluble pectin to a gel by means of pectase, followed by a slow liquefaction of the gel during the progress of the growth of the fungus. This liquefaction is accompanied by an assimilation of at least the furfural-yielding constituents of the pectin. The fungous hyphae contain very little if any furfural-yielding bodies. It is not known whether the enzyme pectinase is involved in the liquefaction of the calcium pectate. Presumably it must be present in order to liberate soluble split products from the gel before the latter becomes available to the hyphae, although this point has not been demonstrated.

HAWKINS, on comparing the analyses of peaches before and after rotting by *Sclerotinia cinerea*, found no appreciable change in the pentosan content. This may or may not be evidence contrary to the preceding. In the whole fruit there is an abundance of carbohydrate food other than the pectin, which the fungus can utilize far more readily. As already stated, growth on the pectin medium was very slow, indicating that the fungus can utilize pectin, but with difficulty; hence it is fair to believe that in the presence of abundant sugars in a natural host the pectins are probably not drawn upon for nutrients.

Secretion of pectase

Whenever *Sclerotinia* is grown on a fruit juice medium containing soluble pectin, the latter is coagulated to a gel. This gel may vary in extent from a few suspended flocs to a solid medium, depending on the amount of pectin present. The gel is insoluble in hot water. It dissolves readily in dilute alkali and in dilute acid, and is reprecipitated from these solutions by alcohol. It is

soluble in ammonium oxalate, with the concomitant formation of crystals of calcium oxalate. In accordance with the usual nomenclature (**2, 5, 10**), therefore, the coagulum is judged to be calcium pectate, and its formation to be brought about by the enzyme pectase, secreted by the fungus. COOLEY demonstrated a similar pectic enzyme, differing from the present one, however, in that it brought about the coagulation in the supposed absence of calcium. He designated his enzyme pectinase, but that is merely a question of nomenclature. As regards the necessity for the presence of calcium, it is possible that the pectin preparation used by COOLEY contained some of this element, brought down during the alcoholic precipitation. He states that a preliminary test of the plum juice with oxalic acid showed the absence of calcium, and that this treatment was therefore abandoned as unnecessary. It is inconceivable that a fruit juice should contain no calcium whatever; hence it is very probable that the pectin coagulated by alcohol did contain some calcium, since the latter is a characteristic constituent of the alcholic precipitate of all plant juices.

In order to determine to what extent pentosans are present in the mycelium of *Sclerotiniа*, either as enmeshed pectic gel or as an actual constituent of the fungous body, a few analyses were made of felts grown on various media. The felts were removed from the media, carefully washed with water, dried between filter paper, and then placed in a desiccator over quicklime. When perfectly dry they were ground to a powder, sampled, and analyzed. The following data were obtained, expressed as percentage of pentosan in the dry mycelium:

No. 568. Grown on synthetic sucrose-salts media entirely free from pectin and pentose; sporulation abundant ..... 3.5 per cent pentosan

No. 569. Grown on whole prune juice, pectin fairly abundant; sporulation moderate ..... 1.5 per cent pentosan

No. 570. Grown on whole peach juice and apricot juice, pectin abundant; sporulation abundant ..... 0.8 per cent pentosan

No. 572. Grown on the prepared pectin medium described; sporulation absent ..... 3.9 per cent pentosan

That the figure obtained in no. 568 actually represents the pentosan content of the mycelia is doubtful. When the phloroglucin was added to the furfural-containing distillate, a bright red

color developed, which gradually changed to a reddish brown precipitate. The normal color change is a green solution at first, changing to a black precipitate. It is possible that the fungus yielded during the acid distillation some substance other than furfural which reacted with the phloroglucin. Furthermore, the media in this case contained no pentose substances whatever; hence it is very unlikely that the fungous bodies would produce more furfural-yielding materials here than in the other cases, where the possibilities of enmeshed pectin were present. The high value for no. 572 is explainable by the fact that the felt grew on a firm gel of calcium pectinate, making it difficult to obtain a clean separation of mycelium from medium in preparing the samples for analysis.

From these data it is evident that only a very small amount of pentosan is present in the felt of *Sclerotinia cinerea*, either as enmeshed pectin or as a constitutional substance. Whether the latter actually exists is not proved by the data. Furthermore, microchemical tests failed to show pectic substances in any portion of the hyphae.

A possible function of the enzyme pectase in a fluid medium, such as fruit juice, is suggested when the characteristics of the growth of *Sclerotinia* on such a medium are taken into consideration. These juices always contain soluble pectin; they also contain some calcium, but there is never any formation of calcium pectate without the presence of the fungous hyphae. As soon as the germinating spores in these media have begun to form a slight weft, this weft can be lifted from the medium with a mass of gelatinous material clinging to it. In fact, the young felt consists of a relatively small mass of hyphae imbedded in a large mass of the gelatinous coagulum. This coagulum can be removed by gentle pressure through cheesecloth in the case of young felts. As they grow older, however, the hyphae increase in bulk, become closely packed together, and the coagulum loses water and becomes firm and slippery. A mature sporulating felt consists typically of three layers: an upper white layer of loosely packed hyphae bearing the sporophores; a middle layer of black, leathery, closely packed mycelium; and a lower layer consisting of a few hyphae and the concentrated

pectin coagulum. The lower layer merges into the loose coagulum of the medium, and most of it easily sloughs off when the felt is washed in water. When an apple juice rather high in pectin is used, the whole mass becomes a rather soft gel within a few days. This gel enables the fungus to build up a semi-solid medium upon which to support aerial hyphae and sporophores.

The possible function of pectase to the fungus when invading a host tissue offers a subject for speculation, at least. The substance of the middle lamella is usually spoken of as calcium pectate. That it is the same substance as the calcium pectate gel formed by pectase is rather doubtful (**2, 5**). The work of various investigators has proved conclusively that this fungus dissolves out the middle lamella, either by the enzyme pectosinase or by oxalic acid. If it be oxalic acid, the calcium of the middle lamella would be removed as insoluble calcium oxalate, leaving a pectin residue, presumably soluble, or at least no longer capable of cementing the cell together. Whether *Sclerotinia* has the power of forming a calcium pectate gel out of such pectic residue is not known, since the nature of this material is not determined. If, however, the solution of the middle lamella be brought about by pectosinase, we have a different case to consider. We really know nothing definite concerning the action of this enzyme on the middle lamella, but presumably some simpler pectic substance would be formed along with a soluble calcium complex. The fungus would penetrate the dissolved substance of the lamella, and then reprecipitate it, presumably as the calcium pectate found in the apple juice cultures. Since it is not known whether the pectin obtained by boiling a ripe fruit in water is the same as the pectin produced by the action of pectosinase on the middle lamella, we are perhaps not entirely justified in assuming that the same substance is formed by pectase acting on dissolved middle lamella *in vivo* and on soluble pectin in an extracted fruit juice. The work of previous investigators, however, gives us good reason to believe that the pectic gels in the two cases are very similar, if not identical (**2, 5, 10**).

Given this hydrophyllic gel, what purpose may it serve the invading parasite? VALLEAU believes that the reason *Sclerotinia*

produces a firm and not a soft rot of plums is that its comparatively large hyphae completely fill the intercellular spaces produced by the collapse of the cells, and that thus the tissue retains its shape and firmness, even after rotting. The writer believes it much more probable that the laying down of considerable calcium pectate gel maintains the firmness of the rotted tissue. This view is further supported by the evidence already stated that this fungus does not consume pectic material in its metabolism to any marked degree.

Certain advantages to the fungus can be seen in this production of calcium pectate gel. Since a fruit, rotted on the tree by this fungus, typically retains its form and turgidity for some time, the fungus is enabled to sporulate (chlamydospores in this case) copiously for several days, during a period when the tree is full of ripe fruit susceptible to the rot. Again, many of these rotted fruits do not drop from the tree; they slowly shrivel up, usually without any break in the skin, and form the characteristic mummies of plum and peach orchards. The following spring these mummies imbibe water readily, due in great part to the strongly hydrophyllic calcium pectate gel, and again sporulate profusely. After this the mummies drop to the ground and remain dormant for a year, when they again imbibe water, and produce an abundance of ascospores at the time of blossoming of the fruit trees.

Summary

Sclerotinia cinerea, when grown on a fruit juice containing soluble pectin, coagulates this pectin to a gel of calcium pectate by means of the enzyme pectase. When simple sugars are available, the fungus does not assimilate pectic substances. When, however, pectin alone is available, it is slowly assimilated. The mycelium contains no pectic substances, except such as occur in particles of calcium pectate gel enmeshed by the hyphal filaments. When the fungus invades a tissue, it follows the line of the middle lamella by dissolving out the latter with the enzyme pectosinase. It probably reprecipitates the pectin of the lamella as calcium pectate. The latter, being a hydrophyllic gel, maintains the firmness of the fruit even after rotting, which is a characteristic of fruit rotted by *Sclerotinia*. This highly imbibing gel is probably also

of service to the fungus at subsequent periods by aiding the organism in acquiring a water supply. The production of pectinase is postulated but not demonstrated.

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LITERATURE CITED

1. Association of Official Agricultural Chemists. Methods of Analysis. Baltimore. 1916.
2. ATKINS, W. R. G., Some recent researches in plant physiology. London and New York. 1916.
3. BEIJERINCK, M. W., and VAN DELDEN, A., Arch. Neerl. Sc. Exact. et Naturell. **29**:418–441. 1903; quoted in citation **10** below.
4. COOLEY, J. S., A study of the physiological relations of *Sclerotinia cinerea* (Bon.) Schröter. Ann. Mo. Bot. Gard. **1**:291–326. 1914.
5. CZAPEK, F., Biochemie der Pflanzen. I. Jena. 1913.
6. HAWKINS, L. A., Some effects of the brown-rot fungus on the composition of the peach. Amer. Jour. Bot. **2**:71–81. 1915.
7. SCHRYVER, S. B., and HAYNES, D., The pectic substances of plants. Biochem. Jour. **10**:539–547. 1916.
8. VALLEAU, W. D., Varietal resistance of plums to brown-rot. Jour. Agric. Res. **5**:365–395. 1915.
9. WILLAMAN, J. J., The function of vitamins in the metabolism of *Sclerotinia cinerea*. Jour. Amer. Chem. Soc. **42**: 549–585. 1920.
10. ZUNZ, E., In Abderhalden's Biochemisches Handlexikon. Vol. 5, s. 562. Berlin. 1911.